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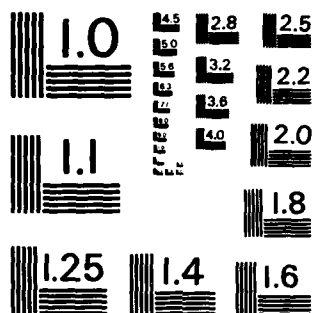
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INSTITUTE REPORT NO. 190

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MUTAGENIC POTENTIAL OF: 4-nitrophenyl dimethyl phosphinate (TA007)
USING THE SEX-LINKED RECESSIVE LETHAL TEST IN *DROSOPHILA MELANOGASTER*

PAUL D. MAUK, BS, SP44
NELSON R. POWERS, PhD, CPT MS
and
ZIA A. MEHR, MS, CPT MS

TOXICOLOGY GROUP
DIVISION OF RESEARCH SUPPORT

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OCTOBER 1984

Toxicology Series 71

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Mutatgenic Potential of: 4-nitrophenyl dimethyl phosphonate (TA007)
using the sex-linked recessive lethal test in Drosophila melanogaster
--Mauk et al

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4-Nitrophenyl dimethyl phosphinate (TA007) has a potential as a prophylactic agent in nerve agent poisoning. Mutagenic activity of this compound was assayed using the Sex-Linked Recessive Lethal test in <u>Drosophila melanogaster</u> . TA007 was determined to be non-mutagenic following 72-hour feeding exposures to 0.01 and 0.005 mM concentrations. ^		

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ABSTRACT

4-Nitrophenyl dimethyl phosphinate (TA007) has potential as a prophylactic agent in nerve agent poisoning. Mutagenic activity of this compound was assayed using the Sex-Linked Recessive Lethal test in Drosophila melanogaster. TA007 was determined to be non-mutagenic following 72-hour feeding exposures to 0.01 and 0.005 mM concentrations.

Key words: Mutagenicity, Toxicology, Sex-Linked Recessive Lethal Test, Drosophila melanogaster, 4 Nitrophenyl Dimethyl Phosphinate

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PREFACE

TYPE REPORT: Drosophila melanogaster Sex-Linked Recessive Lethal Assay

TESTING FACILITY: US Army Medical Research and Development Command
Letterman Army Institute of Research,
Presidio of San Francisco, CA 94129-6800

SPONSOR: US Army Medical Research and Development Command
US Army Medical Institute of Chemical Defense,
Aberdeen Proving Grounds, Aberdeen, MD 21005

PROJECT/WORK UNIT/APC: Medical Defense Against Chemical Agents
35162772A875/Toxicity testing of Phosphinate
Compounds/TL04.

GLP STUDY NUMBER: 82017

STUDY DIRECTOR: COL John T. Fruin, DVM, PhD, VC, Diplomate of
American College of Veterinary Preventive Medicine

PRINCIPAL INVESTIGATOR: CPT Nelson R. Powers, PhD, MS

CO-PRINCIPAL INVESTIGATORS: CPT Zia A. Mehr, MS
SP4 Paul D. Mauk, BS

REPORT AND DATA MANAGEMENT: A copy of the final report, study
protocol, test compound sample, raw
data and SOPs will be retained in
the LAIR Archives.

TEST SUBSTANCE: 4-Nitrophenyl Dimethyl Phosphinate (DMP)
(LAIR Code TA007).

INCLUSIVE STUDY DATES: 10 January 1983 - 6 June 1983

OBJECTIVE: The objective was to assess the mutagenic potential of
the organophosphinate compound 4-nitrophenyl
dimethyl phosphinate (TA007) by using Drosophila
melanogaster in the Sex-Linked Recessive Lethal
Test.

ACKNOWLEDGMENT

The investigators wish to thank SP4 Larry Mullen, BS, for technical advice and Paul Waring, BS, for assistance with the formulation of the test compound. A special debt of gratitude is due Claire N. Lieske, US Army Research Institute of Chemical Defense, who provided test compound, continued advice, and willing inter-agency support.

Signatures of Principal Scientists
Involved in the Study

We the undersigned, believe the GLP Study numbered 82017, described in this report to be scientifically sound and the results and interpretation to be valid. The study was conducted to comply, to the best of our ability, with the Good Laboratory Practice Regulations for Non-clinical Laboratory Studies as outlined by the Food and Drug administration.

John T. Fruin 28 Sep 83
JOHN T. FRUIN, DVM, PhD / DATE
COL, VC
Study Director

Zia A. Mehr 28 Sep 83
ZIA A. MEHR, MS / DATE
CPT, MSC
Co-Principal Investigator

Nelson R. Powers 28 Sep 83
NELSON R. POWERS, PhD / DATE
CPT, MSC
Principal Investigator

Paul D. Mauk 28 Sep 83
PAUL D. MAUK, BS / DATE
SP4, USA
Co-Principal Investigator



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PRESIDIO OF SAN FRANCISCO, CALIFORNIA 94129-6800

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15 Feb 84

MEMORANDUM FOR RECORD

SUBJECT: Report of GLP Compliance

I hereby certify that in relation to LAIR GLP study 82017 the following inspections were made:

11 Jan 83

12 Jan 83

17 Jan 83

22 Feb 83

03 Mar 83

19 Apr 83

11 May 83

27 May 83

The report and raw data for this study were audited on 15 Feb 84.

Routine inspections with no adverse findings are reported quarterly, thus these inspections are also included in the 4 Apr 83 and 5 Jul 83 report to Management and the Study Director.

Lloyd D. Carroll

LLOYD D. CARROLL
1 LT., MSC
Quality Assurance Officer

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MUTAGENIC POTENTIAL OF: 4-Nitrophenyl Dimethyl Phosphinate using the Sex-Linked Recessive Lethal Test in Drosophila melanogaster--
Mauk et al

Organophosphinates are being considered for use as prophylactic agents in nerve agent poisoning. Since the use of these compounds could potentially become widespread, their mutagenicity is being studied. This report contains findings from a Sex-Linked Recessive Lethal (SLRL) mutagenicity test in Drosophila melanogaster of 4-Nitrophenyl Dimethyl Phosphinate.

Rationale for SLRL Testing

A variety of tests using Drosophila are available for the detection of specific types of genetic changes. The most sensitive assay which detects the broadest range of mutations is the SLRL test (1-3). This test uses insects of a known genotype and detects lethal mutagenic changes in 800 to 1000 loci on the X-chromosome, representing 80% of the X-chromosome or 20% of the entire genome (4,5). The SLRL test has been used in most of the research on the mutagenic response of Drosophila to test substances (1,3,4).

Genetic Basis of the SLRL Test

The basic mechanism of the SLRL test is that the X-chromosome of the father is passed onto the daughter; the sons receive their X-chromosome from the mother. The recessive lethal mutations located on the X-chromosomes are expressed in males in a hemizygous condition, and since the Y-chromosome does not contain the dominant, wild-type alleles to suppress their manifestation, this results in death.

The SLRL test is based on the fact that among the progeny of females carrying a recessive lethal mutation on one of her X-chromosomes (heterozygous for a recessive lethal mutation), half of the sons die. By using suitable genetic markers, the class of males carrying the X-chromosomes of treated grandfathers can easily be determined. If a lethal mutation were induced, this class would be

missing and its absence easily scored. This test is also called the Basc or Muller-5 test (5,6). The test system uses strains in which crossing-over in females is prevented since transfer of the lethal mutation from the paternal to the maternal X-chromosome by genetic recombination would suppress its expression. This crossing over would lead to erroneous study results because males receiving that X-chromosome would then survive. Combinations of suitable inversions effectively inhibit crossing over, females used for the test carry two scute inversions: the left-hand part of sc^{S1} and the right-hand part of sc^8 covering the whole X-chromosome and a smaller inversion, In-S, in the Basc chromosome (5).

Description of Test

The SLRL test (7) was developed in 1948 for determining genetic changes which in the hemizygous, but not homozygous or heterozygous, condition kill the developing individual (egg to pre-adult stage). Such genetic changes, i.e. recessive lethal mutations, can be induced on all chromosomes. Only two test generations are needed to detect whether sex-linked recessive lethal mutations have been induced on the X-chromosome.

In the test, wild-type males with normal round red eyes (Canton-S (CS)), are exposed to the phosphinate. Such an exposure will produce a recessive lethal mutation if the X-chromosome is affected. These males are mated to homozygous Basc females. The Basc phenotype is characterized by bar (narrow-shaped) eyes which are white-apricot in color. The bar eyes serve as a genetic marker for the homozygous and hemizygous genotypes since in the heterozygous expression the eyes are kidney-shaped. The progeny of this cross now consists of females heterozygous for the treated X-chromosome, characterized by kidney-shaped red eyes and males of the Basc phenotype that have received their X-chromosome from their Basc mother. Each F_1 female possesses one paternal X-chromosome which was exposed to test compound in the male gamete. F_1 siblings are allowed to mate producing the F_2 generation. The F_2 generation will consist of males of two phenotypic expressions and females of two phenotypic expressions. The male phenotypes are rounded eyes (heterozygous, carrying the treated x-chromosome from the F_1 female) and bar-shaped apricot eyes (hemizygous for the Basc chromosome). The female phenotypes are kidney-shaped red eyes (heterozygous, carrying the treated x-chromosome from the F_1 females and the Basc chromosome) and bar-shaped apricot eyes (homozygous for the Basc chromosome). The F_2 generation is then inspected for the presence of males with round red eyes. If this class is missing, it can be concluded that the treated male gamete contained a recessive lethal mutation. Thus, this test relies upon the disappearance of a whole Mendelian class (males with round, red eyes).

A brooding technique was used to sample sperm cells exposed to the test chemical during different stages of spermatogenesis because chemicals often exhibit stage specific mutagenicity. Brooding was accomplished by transferring the treated males to vials containing fresh virgin females at intervals of 1, 4, 6, and 8 days after completion of the dosing period. This technique assures that the four broods of females are inseminated with sperm exposed to the test chemical during successive stages of germ cell development: Brood 1 = mature sperm (Days 1-3); Brood 2 = primarily spermatids (Days 4-5); Brood 3 = primarily meiotic stages (Days 6-7); and Brood 4 = primarily spermatogonia (Days 8-10). This procedure safeguards against the possibility that chemicals with more pronounced effects in earlier stages of spermatogenesis are not dismissed erroneously as false negatives.

Objective of the Study

The objective was to assess the mutagenic potential of the organophosphate compound 4-nitrophenyl dimethyl phosphinate (TA007) by using Drosophila melaogaster in the Sex-Linked Recessive Lethal Test.

MATERIALS AND CONDITIONS

Test Substance

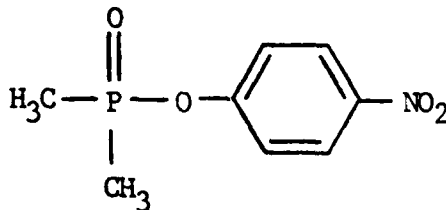
Chemical name: 4-Nitrophenyl Dimethyl Phosphinate

Lot number: XXXXII-67

LAIR Code: TA007

Chemical Abstract Service Registry Number: 13344-08-6

Molecular structure:



Empirical formula: $C_8H_{10}NO_4P$

Molecular weight: 215.15

Purity: unknown

Vehicle

A mixture of 5.0 mM citrate buffer plus 1% fructose was found during the pilot study to be most suitable for use as the vehicle for TA007.

Chemical Data are listed in Appendix A.

Test Model

Insect Genus and Species: Drosophila melanogaster

Strains: Canton-S (CS), a wild-type stock, characterized by round-red eyes, was selected for mutagenicity studies because it has shown a relatively low constant spontaneous mutation frequency (8).

Basic, a laboratory stock, homozygous in females, possesses bar eyes, apricot-colored eyes, and scute as phenotypic markers. The genetic designation is $in(1)sc^{SL}sc^{BR}in(1)S, sc^8, sc^{SL}w^{AB}$.

Both strains are presently being reared in the insectary at Letterman Army Institute of Research. The original stock colonies were obtained from the Mid American Drosophila Stock Center, Bowling Green State University, Bowling Green, Ohio.

Diet

The diet was the standard medium consisting of cornmeal, unsulfured molasses, yeast, and nutrient agar used for colony rearing of D. melanogaster. A materials list and instructions for its preparation are contained in LAIR SOP-OP-STX-5 Drosophila Media Preparation.

Restraint

Ether anesthesia was used for restraint of flies being collected for mating and for general colony maintenance.

Identification System

Each CS male from the 72-hour LC_{50} exposure (test, negative, positive control) had a unique number assigned and placed on the vial in which its progeny was produced (LAIR SOP-OP-STX-8 "Sex-Linked Recessive Lethal (SLRL) Drosophila melanogaster Mutagenicity Test"). In this manner progeny were traced back to the parental male which had been subjected to the test compound or controls.

Environmental Conditions

All studies were conducted within the insectary at a temperature of 21 ± 4 C, relative humidity of $50 \pm 5\%$ and a photoperiod of 12 hours light and 12 hours dark. All insect colonies were reared in polypropylene bottles and SLRL testing was done in glass vials (LAIR SOP-OP-STX-6 "Drosophila Stock Colony Maintenance").

Dosing

Test compound formulation was done in accordance with LAIR SOP-OP-STX-64 "Preparation of Additional Compounds Unstable in Water for SLRL Assay" and dosing was accomplished in compliance with LAIR SOP-OP-STX-7 "Drosophila melanogaster Exposure Procedures". The test compound solutions were checked daily during the dosing periods for hydrolysis using a spectrophotometer. The CS strain (wild-type) males were allowed to feed on 250 μ l of various concentrations of the test chemical formulated with 5.0 mM citrate buffer and 1% fructose in water. These males formed the test groups. Concurrent exposure to 5.0 mM citrate buffer and 1% fructose in water was designated as the negative control group. A positive control group was exposed to a 1 mM ethylmethane sulfonate solution formulated with 5 mM citrate buffer and 1% fructose. Ethylmethane sulfonate is a known mutagen and was used to confirm the ability of the test organism to produce SLRL mutations (9). Dosing was continuous for 72 hours. Flies were transferred every 24 hours to vials containing fresh compounds. A pilot toxicity study was conducted to determine the upper and lower limits of mortality before LC_{50} determination. The LC_{50} (approximate) for 72 hours was the dosage used in mutagenicity testing (4). The dosages tested for toxicity were 1.0, 0.1, 0.05, 0.01, 0.005, and 0.001 mM dimethyl phosphinate. The LC_{50} determination was conducted 4 times, once for each replicate.

Test Format

The CS males treated with a LC_{50} (approximate) of 4-nitrophenyl dimethyl phosphinate and those males subjected to the concurrent negative controls were used in the SLRL assay. Survivors from the test chemical and negative control compound were scored by mating 25 dosed CS males (wild-type) to Basc virgin females (Basc chromosome). This was done by placing 3 Basc virgin females in a vial with one CS male, that vial being labeled with the male's unique number. At days 1, 4, 6, and 8 after dosing the CS male was transferred to successive groups of 3 Basc virgin females in vials with that male's unique number. These intervals of days corresponded to broods 1, 2, 3 and 4. This procedure was replicated 4 times. Scoring of the mutants resulting from positive control exposure was based on mating 5 CS males in the same manner as males treated with the test compound.

This was replicated 4 times. After sufficient numbers of flies had emerged, a maximum of 25 (minimum of 5) kidney-shaped red-eyed F_1 females were selected at random and mated with their sibling bar-shaped apricot eyed males. Each pair was placed in an individual vial, and these vials from the same unique numbered father were placed together and labeled with that unique number for reference. After 2 to 3 weeks the F_2 progeny were examined and scored for the absence of round red-eyed males, which would indicate that a lethal mutation had taken place in the treated male. Confirmation of a lethal mutation was obtained by conducting a F_3 cross from each vial scored as a lethal mutation. This was accomplished by crossing three F_2 females (kidney-shaped red-eyes) with one male with bar-shaped apricot eyes. Absence of males with round red eyes in the resulting F_3 generation confirmed the existence of a recessive lethal mutation. Experimental conclusions were based on the spontaneous mutation frequency (negative control) compared to the mutation frequency induced by the test chemical.

Historical Listing of Significant Study Events

Appendix B is a historical listing of study events.

Statistical Analysis

This testing was designed to examine 2500 X-chromosomes in each of 4 replications thereby yielding a total of 8000 to 10,000 X-chromosomes for examination. The mutation frequency of the phosphinate was compared to that of the negative control (spontaneous mutation frequency) by means of the Fisher's exact test using a 2 x 2 table (10, 11) a more conservative test, the Kastenbaum-Bowman test (12, 13, 14) was also considered. Both tests were based on the number of lethal and non-lethal culture vials of the total number examined for each unique numbered male (control and treated).

Each culture vial contained F_2 progeny and is regarded as an X-chromosome (5). Vials without F_2 progeny or less than 5 progeny (F_2) were scored as failures. In addition, the mutation frequency from each of the four broods was also analyzed using Fisher's Exact Test.

Deviations from Procedure during Study

The following deviations from the Standard Operating Procedures were made in the study:

Fructose was omitted from the 1.0 mM preparation of test compound formulated for use in the 72-hour LC_{50} determination for Replicate One (Run 45). This error was corrected at 24 hours after dosing and did not affect the outcome of the LC_{50} (approximate).

The total number of flies examined was below the stated minimum of 8000; however, 7980 flies were examined from the Negative Control group and 7567 from the Test group. This was considered an adequate sample size for statistical analyses using Fisher's Exact and the Kastenbaum-Bowman tests.

RESULTS

The percent mortalities of the concentrations of TA007 that most closely approximated the LC_{50} after a 72-hour exposure are shown for each replication in Table 1. These concentrations were selected for use in the appropriate replication because they gave the closest approximation to the LC_{50} of the concentration used in the pilot study.

The mutation frequencies for TA007 and the negative control were 0.225% and 0.163%, respectively. The mutation frequency for the positive control, 1 mM ethyl methane sulfonate, was 14.878%. These data are displayed in Table 2. The mutation frequencies for each brood for test chemical exposure and the negative control are shown in Table 3. Tabular data from this study (GLP 82017) for each male are in the archives of Letterman Army Institute of Research, Presidio of San Francisco, California.

TABLE 1

Concentrations and Corresponding Mean Percentage Mortality and Standard Deviation for TA007* fed to CS Males for the SLRL Assay.

Replication Number	Concentration mM	%Mortality x + s.d. [†]
1	0.01	68.0 + 18.7
2	0.005	59.0 + 20.2
3	0.005	53.6 + 28.6
4	0.005	66.0 + 30.6

*TA007 = 4-nitrophenyl dimethyl phosphinate formulated with citrate buffer (5.0 mM) and 1% fructose in H₂O.

[†]Based on a sample size ranging from 95 to 100 CS males.

TABLE 2

Sex-Linked Recessive Lethal
Assay of TA007

Compound	Replication*				Total	%Mutation
	1	2	3	4		
TA007†	2/1695	3/2004	7/1883	5/1985	17/7567	0.2247
Negative Control‡	2/1845	4/1939	5/2226	2/1970	13/7980	0.1629
Positive Control"	35/267	45/265	50/280	28/250	158/1062	14.878

*Data are recorded as number of SLRL events/number of X-Chromosomes tested.

†TA007 = 4-nitrophenyl dimethyl phosphinate formulated with 5.0 mM citrate buffer and 1% fructose. 25 male *D. melanogaster* flies (CS strain) formed the P generation.

‡Negative Control = 5.0 mM citrate buffer and 1% fructose.
25 male *D. melanogaster* flies (CS strain)
formed the P generation.

"Positive Control = 1.0 mM ethylmethane sulfonate and 1% fructose
5 male *D. melanogaster* flies (CS strain)
formed the P generation.

TABLE 3

Sex-Linked Recessive Lethal Assay
for Each Brood of TA007

Compound	Brood*			
	1	2	3	4
TA007†	5/1990	4/2073	6/1794	2/1693
Negative Control‡	7/2212	5/2207	0/2036	1/1512
Positive Controls"	48/364	56/372	52/304	2/22
p values (> 0.05) Fisher's Exact	0.4602	0.5385	0.0105	0.5423

*Data are recorded as number of SLRL events/number of X-chromosomes tested. Data were pooled from 4 replicates for each brood.

†TA007 = 4-nitrophenyl dimethyl phosphinate formulated with 5.0 mM citrate buffer and 1% fructose. Data are from 25 male *D. melanogaster* flies (CS strain) x 4 replicates mated with 3 Basc strain female flies each.

‡Negative Control = 5.0 mM citrate buffer and 1% fructose. Data are from 25 *D. melanogaster* flies (CS strain) x 4 replicates mated with 3 Basc strain female flies each.

"Positive Control = 1.0 mM ethylmethane sulfonate and 1% fructose. Data are from 5 male *D. melanogaster* flies (CS strain) x 4 replicates mated with 3 Basc strain female flies each.

DISCUSSION

The spontaneous mutation frequency was 0.163% based on 7980 X-chromosomes, while the mutation frequency resulting from TA007 was 0.225% based on 7567 X-chromosomes (Table 2). Detection of a doubling in this spontaneous mutation frequency (evidence of a weak mutagen) would require a minimum of 7000 X-chromosomes to be examined, based on the tables given in Kastenbaum and Bowman (13, 14). Therefore, our sample size was considered adequate for the Kastenbaum-Bowman analysis.

The results of the Fisher's exact test using a 2 x 2 table implemented by use of the Bio-Medical Programs, 4F Program (12), indicated there was no-significant difference between the mutation frequency of the negative control and TA007; the p-value (0.2439) for a one-tailed test was non-significant at the 5% level. The one-tailed Kastenbaum-Bowman test revealed non-significant difference between TA007 and the negative control at the 5% level ($p = 0.2449$), ($m = 30$, $k = 0.487$).

Analysis of the pooled data for Broods 1, 2, 3, and 4 (Table 3) indicated that differences between the negative control and test compound are non-significant except for Brood 3. This would suggest that the compound was mutagenic during the meiotic stage of spermatogenesis. However, this significant observation can be attributed to the statistical anomaly that no lethal mutations were observed in the Brood 3 negative controls. This conclusion is supported by the observation that the mutation rate in the Brood 3 phosphinate test group was no different from the mutation rate for negative control broods 1 and 2 and considerably lower (1/50th) than the mutation rate in the positive control.

CONCLUSION

The results of this study indicate that 4-nitrophenyl dimethyl phosphinate is not mutagenic when evaluated in the Drosophila melanogaster Sex-Linked Recessive Lethal Assay.

RECOMMENDATION

Additional testing of 4-nitrophenyl dimethyl phosphinate is not recommended at this time.

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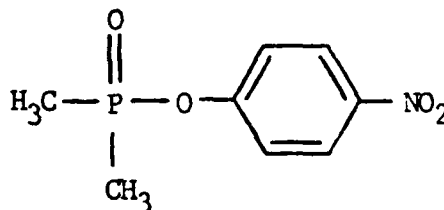
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APPENDICIES

Analytical and Physical Chemical Data

Chemical name: 4-Nitrophenyl Dimethyl Phosphinate

Molecular structure:



Chemical Abstract Service Registry Number: 13344-08-6

Empirical formula: C₈H₁₀NO₄P

Molecular weight: 215.15

pH: N/A non-aqueous

Physical state: solid crystals

Boiling point: N/A

Melting point: unknown

Compound refractory: N/A

Stability: Under refrigerated conditions, Dr. Lieske (Biomedical) Laboratory, Aberdeen Proving Grounds, Aberdeen MD, 21005) believed the compound would remain stable for two years.

Names of contaminants and percentages: unknown

Manufacturer: Ash Stevens
Detroit Research Park
5861 John C. Lodge Freeway
Detroit, Michigan 48202

Manufacturers Lot No.: XXXXII-67

This sample was kept from exposure to light and frozen, as required.

Analytical data: 4-Nitrophenyl dimethyl phosphinate
formulated with citrate buffer and water.

The various concentrations of 4-nitrophenyl dimethyl phosphinate (DMP) (TA007) were prepared as follows:

10 mM 4-nitrophenyl DMP stock solution [0.0100g DMP, 10 ml 5.0 mM citrate buffer].

1.0 mM 4-nitrophenyl DMP stock solution [1 ml of 10 mM DMP, 9 ml of 5.0 mM citrate buffer]. 1.0 mM DMP solution [1 ml of 1.0 mM DMP, 8 ml of 5.0 mM citrate buffer, 1 ml of 10% fructose in citrate buffer].

0.1 mM DMP solution [2 ml of 10 mM DMP, 16 ml of 5.0 mM citrate, 2 ml 10% fructose in citrate buffer].

0.05 mM DMP solution [5 ml of 0.1 mM DMP, 4 ml of 5.0 mM citrate, 1 ml 10% fructose in citrate].

0.01 mM DMP solution [1 ml of 0.1 mM DMP, 8 ml of 5.0 mM citrate, 1 ml of 10% fructose in citrate].

0.005 mM DMP solution [1 ml of 0.05 mM DMP, 8 ml of 5.0 mM citrate, 1 ml of 10% fructose in citrate].

0.001 mM DMP solution [1 ml of 0.01 mM DMP, 8 ml of 5.0 mM citrate, 1 ml of 10% fructose in citrate].

The negative control was prepared with the 5.0 mM citrate buffer and 10% fructose in citrate buffer. The final concentration of fructose was made to equal 1%.

Physical state: clear aqueous solution

pH: 4.0

Stability: Hydrolysis measurements were conducted immediately after preparation, at 24 hours, 48 hours, and 72 hours. 4-Nitrophenyl DMP hydrolyzed at a rate of about 10% per 24 hours at the 10 mM concentration when refrigerated.

Historical Listing of Significant Study Events

3-7 Jun 82	4-Nitrophenyl dimethyl phosphinate (TA007) formulated according to LAIR SOP-OP-STX-64, Preparation of Additional Compounds Unstable in Water for SLRL Assay, for pilot toxicity testing in <i>Drosophila melanogaster</i> .
10 Jan 83	Removal of all adult insects from CS colony and collecting of newly emerged CS males 24 hours later.
11 Jan 83	4-Nitrophenyl dimethyl phosphinate (TA007) prepared according to LAIR SOP-OP-STX-64. Dosing of newly emerged CS males begins.
12-13 Jan 83	CS males transferred to freshly prepared dosing tubes. Flies surviving 72-hour LC determination are placed with 3 virgin Basc females and given an identification number. Begin first replicate (Run 45).
24-28 Jan 83	Begin Replicate 2 (Run 46).
28-31 Mar 83	Begin Replicate 3 (Run 47).
18-21 Apr 83	Begin Replicate 4 (Run 48).
1 Feb - 6 Jun 83	The F crosses of all broods for all 4 replicates were made and scored as were the F s.

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